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Resident Microbiota of the Gypsy Moth Midgut Harbors Antibiotic Resistance Determinants

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Little is known about the significance of insects as environmental reservoirs of antibiotic-resistant bacteria. We characterized the antibiotic resistome of the microbial community in gypsy moth larval midguts by applying functional metagenomics to cultured isolates. The minimum inhibitory concentrations of 12 antibiotics were determined for 44 cultured isolates, and antibiotic resistance genes were selected from metagenomic libraries derived from DNA extracted from a pool of the isolates. Six unique clones were identified. Two were highly resistant to penicillin-type β-lactams, two were moderately resistant to erythromycin, and two were moderately resistant to a range of antibiotics, including erythromycin, carbenicillin, and chloramphenicol. Sequence analysis predicted that the active genes encoded efflux pumps, a transcriptional activator of efflux pump protein expression, and an extended-spectrum class A β-lactamase. Insect guts are a reservoir of antibiotic resistance genes with the potential for dissemination.

Introduction

Insects comprise one of the most diverse taxa of life, and it is therefore surprising that little is known about their commensal bacteria and the impact of these bacteria on the health of their insect hosts. Even less is known about the implications of insect-associated bacteria on human health. For example, despite the importance of antibiotic-resistant bacteria in modern medicine, only a few insect species have been screened for them. Significantly, antibiotic-resistant human pathogens are carried by flies and cockroaches in hospitals and other urban settings (Pai *et al.*, 2004, 2005; Rahuma *et al.*, 2005; Macovei and Zurek, 2006). The insect gut may also serve as a mixing ground for bacterial genes. Antibiotic resistance genes transfer conjugally from *Escherichia coli* to *Yersinia pestis*, the causal agent of the Black Plague, within its flea host (Hinnebusch *et al.*, 2002). Insects may also apply selection pressures for antibiotic resistance through their diet. Oil fly larvae, for example, that had no known exposure to antibiotics carry antibiotic-resistant bacteria that might be selected from among commensal bacteria by a general cell stress response incited by solvent exposure (Kadavy *et al.*, 2000). Finally, as highly successful invasive organisms, insects present a convenient distribution mechanism for antibiotic-resistant bacteria. When an insect invades a new environment, its resident microbiota is carried along and may invade as well (Vasanthakumar *et al.*, 2008).

The gypsy moth (Lepidoptera: *Lymantria dispar*) is an interesting example of an invasive species that may provide a number of selective pressures for antibiotic resistance on its resident microbiota. The microbial community of the gypsy moth midgut is dominated by *Enterococcus* spp. and members of the Enterobacteriaceae, which are commensal species of humans (Broderick *et al.*, 2004). Antibiotic resistance has been increasing in clinical isolates of these taxa, presenting a significant barrier to successful treatment of infectious diseases (Paterson, 2006; Rice, 2006). Some members of these groups have been shown to be efficient in gene exchange in gut environments (Salyers *et al.*, 2004). Gypsy moth larvae consume a wide range of host plant species (Stoyenoff *et al.*, 1994), exposing its midgut microbial community to diverse plant compounds, some of which may resemble antibiotics. In addition, the gypsy moth midgut has a pH up to 12.4, which may impose an additional selection pressure (Gringorten *et al.*, 1993). The gypsy moth midgut microbiota therefore encounters a wide range of selection pressures.

Metagenomics, the collective analysis of an assemblage of organisms, was developed to study the genome of an entire microbial community (Riesenfeld *et al.*, 2004). In soil, for example, less than 1% of the bacteria are culturable by standard laboratory techniques (Torsvik *et al.*, 1990; Amann *et al.*, 1995); therefore, metagenomics provides a tool to access the majority of genes in the community. Metagenomics has not, however, been applied to assemblages of cultured

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organisms. We studied the antibiotic resistance phenotypes of 44 bacterial isolates from gypsy moth midguts, using metagenomics as an expedient way to access the genotypes responsible for resistance. Here we report the application of metagenomics as an efficient means to investigate the genetic basis for antibiotic resistance in 44 bacterial isolates, describe three new antibiotic resistance genes, and identify the bacteria from which the genes were isolated.

Materials and Methods

Gypsy moth bacterial isolates

Thirty-four bacterial isolates representing 15 species were previously isolated from the midguts of lab-reared gypsy moth larvae feeding on diverse plants (Broderick *et al.*, 2004) (Table 1). For the current study, additional wild, third and fourth instar gypsy moth larvae were collected from Cross Plains and Manitowoc, Wisconsin, by the Forest Pests Unit of the Wisconsin Department of Natural Resources. These larvae were stored less than 12 h at room temperature with the foliage on which they were collected in closed, insulated containers until dissection.

The midguts of 60 wild larvae were dissected and suspended in 100 mL phosphate buffer (35 mM KH_2PO_4, 15.5 mM Na_2PO_4, pH 7). Dilutions of the midgut suspension were cultured immediately on 1/10th strength tryptic soy agar (10% TSA), and then the gut suspension was incubated overnight at 28°C and cultured again in the same manner. All 10% TSA plates were incubated for 48 h at 28°C. A single colony of each morphology was cultured for purity, yielding a total of 10 bacterial isolates from wild gypsy moth midguts. Isolates were stored in 1/10th strength tryptic soy broth (10% TSB) containing 10% glycerol at −80°C.

The 16S rRNA gene of each isolate was PCR amplified using the primers 27F (5′ AGRGTTTGATYMTGGCTCAG 3′) and 1492R (5′ GGYTACCTTGTTACGACTT 3′) at an annealing temperature of 55°C. PCR products were purified with Ampure (Agencourt Bioscience, Beverly, MA) according to the manufacturer's instructions, and sequenced with 27F and 1492R and Big Dye 3.1 (Applied Biosystems, Foster City, CA). Reaction products were purified with CleanSeq (Agencourt Bioscience) following the manufacturer's instructions. Results were analyzed on Applied Biosystems 3730×1 sequencers at the University of Wisconsin-Madison Sequencing Facility. Sequences were compared to those in GenBank with BLAST (Altschul *et al.*, 1990, 1997). 16S rRNA gene libraries were also constructed from the 60-midgut suspension following the amplification protocol above. PCR products were cloned into pGEM-T (Promega, Madison, WI), inserts were amplified with vector primers SP6 and T7, and amplification products were sequenced with primer 27F as indicated above.

Determination of minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) for carbenicillin (Fisher Scientific, Fair Lawn, NJ), erythromycin (Fluka BioChemika, Buchs, Switzerland), ciprofloxacin (Wako Chemicals USA, Richmond, VA), rifampin (Research Products International, Mt. Prospect, IL), ceftazidime, gentamicin, kanamycin, streptomycin, vancomycin, chloramphenicol, nalidixic acid, and tetracycline (all from Sigma, St. Louis, MO) were determined by broth microdilution for each isolate according to the Clinical Laboratory Standards Institute (CLSI) guidelines (NCCLS, 2004). Briefly, each isolate was grown in 1/2-strength tryptic soy broth (50% TSB) overnight at 28°C. The culture density was adjusted to approximately 1×10^8 colony forming units (CFU) per mL in Mueller–Hinton broth (Becton, Dickinson and Company, Sparks, MD) based on optical density at 600 nm. Each suspension was diluted in Mueller–Hinton broth 1:20 and 10 μL was inoculated into 100 μL Mueller–Hinton broth containing a dilution series (512–0.5 μg/mL) of the test antibiotic in 96-well plates (Corning Costar, Corning, NY). The concentration and purity of each isolate was verified by dilution plating on 1/10th-strength TSA (10% TSA). Both the 96-well and 10% TSA plates were incubated for 16–18 h at 28°C.

As per CLSI guidelines, the MIC was recorded as the lowest concentration of antibiotic to prevent turbid growth based on visual assessment and comparison to uninoculated wells, inoculated wells containing only Mueller–Hinton broth, and each antibiotic dilution series inoculated with EPI300 *E. coli*. The results reported for EPI300 *E. coli* fall within the accepted range of MIC values for *E. coli* (Table 3 and data not shown) (NCCLS, 2004). MIC values were measured at twofold increases in concentration; therefore, apparent differences appear more significant at higher concentrations, and minor differences are likely attributable to differences in gene expression levels and experimental variation. The MIC values were confirmed in at least three separate experiments, and as long as the values were within two dilution factors, the lowest value obtained for each isolate was reported. Although clinical guidelines were used for antibiotic resistance testing, clinical standards do not exist for most environmental bacteria, including those from the gypsy moth midgut. Therefore, an isolate was called "resistant" if it showed more than twofold higher resistance compared to other gypsy moth isolates of the same species. MIC assays for metagenomic clones were performed as described except that *E. coli* cultures were grown in LB at 37°C. MIC assays were also performed on a subset of clones with the β-lactam antibiotics amoxicillin and piperacillin (Sigma). β-Lactamase inhibitor assays were performed in a similar manner except that 2 μg/mL potassium clavulanate (Sigma) or 4 μg/mL tazobactam (Sigma) were added to each test well.

Metagenomic library construction from pooled isolates

DNA extractions of gypsy moth midguts were unsuccessful; therefore, metagenomic libraries were built from the cultured members of the community. Bacterial isolates were struck out from freezer stocks onto 10% TSA and incubated for 48 h at 28°C. Because metagenomic library construction necessitates more DNA than is isolated from a single colony, single colonies were swabbed onto 10% TSA plates and incubated for 48 h at 28°C. These bacterial lawns were scraped into pools of six isolates for DNA extraction via the PowerSoil DNA Extraction Kit (MOBIO Laboratories, Carlsbad, CA). The pools were necessary to fit the volume of the tubes supplied with the PowerSoil Kit, and bacterial DNA from all isolates was combined prior to digestion. DNA was partially digested with *Pst*I and ligated into pCF430 (Table 2) (Newman and Fuqua, 1999). Ligation products were transformed into *E. coli* EPI300 (Table 2) (Epicentre Biotechnologies,

Madison, WI), and the resulting colonies were stored in pools in LB containing 10% glycerol. Two metagenomic libraries, GMC01 and GMC02, were constructed in this manner.

Selection and phenotypic confirmation of antibiotic-resistant clones

Libraries GMC01 and GMC02 were selected on LB containing 20 mM arabinose and 50 μg/mL carbenicillin, 5 μg/mL ceftazidime, 20 μg/mL chloramphenicol, 1 μg/mL ciprofloxacin, 10 μg/mL gentamicin, 500 μg/mL erythromycin, 20 μg/mL kanamycin, 50 μg/mL nalidixic acid, or 30 μg/mL rifampin. One hundred microliters of a suspension of approximately 1×10^6 CFU/mL of each library was spread on eight plates of each selective medium. One set of four plates was incubated overnight at 37°C and the other set at 28°C. Two aliquots of each library were selected independently. Plasmid DNA from each presumptive antibiotic-resistant clone was isolated by alkaline lysis (Sambrook and Russell, 2001). DNA from each clone with a unique restriction pattern was retransformed into *E. coli*, and the antibiotic resistance phenotype and plasmid restriction patterns were confirmed. Metagenomic clones are listed in Table 2.

Identification and DNA sequencing of antibiotic resistance genes

The genes responsible for the resistance phenotype in clones ERY01 and CRB03 were determined by mutagenesis with the GPS-1 genome priming system as per manufacturer's instructions (New England Biolabs, Ipswich, MA). Loss-of-phenotype mutants were identified by plating transformants onto permissive (LB + 20 μg/mL tetracycline + 20 μg/mL chloramphenicol) and selective media (LB + 500 μg/mL erythromycin or 50 μg/mL carbenicillin), and identifying colonies that could no longer grow under selection. Plasmid DNA was isolated from these colonies, and the active gene was sequenced using the north and south primers provided with the transposon (New England Biolabs). Additional primers (IDT, Coralville, IA) were designed to sequence the entire insert of ERY01.

Clone CRB01 was partially digested with *Sau*3aI (Promega) and purified using the PCR clean-up protocol in the QIAquick gel extraction kit (Qiagen, Valencia, CA). The resulting DNA fragments were ligated into the *Bam*HI site of pET24a (Novagen, Madison, WI), and the ligation was transformed into EPI300 *E. coli* (Epicentre Biotechnologies). Carbenicillin-resistant subclones were selected, and one containing a 3.3-kb insert was sequenced by primer walking. Sequences were assembled in SeqMan (DNAstar, Madison, WI). ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to identify putative open reading frames (ORFs), which were annotated using BLAST (Altschul *et al.*, 1990, 1997). Annotation revealed a gene encoding a β-lactamase, and its function was verified by cloning it into the *Xho*I and *Nhe*I sites of pET24a (Novagen). Primers were designed with appropriate restriction sites (forward, 5′-atctgctagcATGAATAGCATCTTTTCCCTGTCC; reverse, 5′-aattctcgagTTACTGTTTTTCCCACGCCGCCAG; capital letters indicate coding region, and underlining indicates restriction sites) (IDT), and the PCR product was digested and ligated into pET24a. The ligation products were transformed into EPI300 *E. coli*, carbenicillin-resistant clones were selected, and the construct was designated LRG1 (Table 2). One resistant clone was sequenced and tested in MIC assays. *ramA* was cloned from CRB03 (forward primer, 5′-gactgctagcATGATAAAAGGAGAGGCAGCAAT; reverse primer, 5′-atatctcgagCTACGCCCGAACCTGATGCTCT) (IDT) and tested for resistance in the same manner as $bla_{\text{LRG-1}}$. This construct was called RAMA (Table 2).

To identify the organism of origin for the metagenomic resistance genes, bacterial colonies of each isolate were suspended in 50 μL colony lysis solution (CLS) (1% Triton X 100, 20 mM Tris-Cl pH 8.5, and 2 mM EDTA) and boiled for 20 min. PCR reactions were carried out in 50-μL reaction mixtures containing 5 μL bacterial lysate, GoTaq DNA polymerase and buffer (Promega), 1 mM dNTPs (0.25 mM each), and 25 pmol of each primer. The following primer pairs were designed to probe the bacterial isolates for the resistance genes found in the metagenome: sdeXf (5′-ATGAACAAAAACAGAGGGTTAACG) and sdeXr (5′-TTATGACTTCTGCGCTTCAGACTG); ramAf (5′-ATGATAAAAGGAGAGGCAGCAAT) and ramAr (5′-CTACGCCCGAACCTGATG); lrg1f (5′-ATGAATAGCATCTTTTCCCTGTCC) and lrg1r (5′-TTACTGTTTTTCCCACGCCGCCAG) (IDT). *ramA* and *lrg1* were amplified with the following thermal cycle: 95°C for 3 min, 30 cycles of (denature [94°C for 30 s], extend [53°C for 1 min], and anneal [72°C for 2 min]), 72°C for 7 min, and 4°C hold. *sdeX* was amplified with the following thermal cycle: 95°C for 3 min, 30 cycles of (denature [94°C for 30 s], extend [49°C for 1 min], and anneal [72°C for 2 min]), 72°C for 7 min, and 4°C hold. Five microliters of CLS was subjected to PCR amplification as a negative control, and 1 μL of plasmid preps of ERY01 (with the *sdeX* primer pair), CRB03 (with the *ramA* primer pair), and CRB01 (with the *lrg1* primer pair) was always subjected to amplification as a positive control. Five microliters of each PCR reaction was run on an agarose gel and visualized with ethidium bromide staining.

Phylogenetic analysis of LRG-1

The amino acid sequences of the top 100 homologs to LRG-1 were retrieved via BLAST (Altschul *et al.*, 1990, 1997). To improve readability of the phylogenetic tree for publication, some sequences were eliminated from dense clades before the final analysis (e.g., not all CTX-M β-lactamases are presented). Sequences were aligned in ClustalX 1.83 (Thompson *et al.*, 1997) with the following parameters: pairwise gap opening penalty = 35, pairwise gap extension penalty = 0.75, multiple gap opening penalty = 15, and multiple gap extension penalty = 0.3. The alignment was optimized by trimming < 5 amino acids from either end of the alignment, and by hiding a gap near amino acid 40. The alignment was then analyzed by neighbor-joining and maximum parsimony (MP) in Paup*4.0b10 [(Swofford, 2003) 1000 bootstrap replicates], and in Bayesian analyses in MrBayes 3.1 [(Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) 200,000 MCMC generations, burnin of 200]. Prottest (Abascal *et al.*, 2005) determined that the fixed-rate model WAG + G [(Whelan and Goldman, 2001), general reversible model of amino acid replacement and γ-distributed rates] best fit the data under the BIC framework. The Bayesian tree is reported, and is rooted with the chromosomal β-lactamase from *Erwinia perscina*, ERP-1. The topology of the parsimony tree conformed to the Bayesian tree except at nodes where no bootstrap value is reported, in which case the Bayesian tree provided better resolution.

GenBank accessions

The metagenomic clones that were sequenced were deposited in GenBank, and their accession numbers are listed in Table 2. The 16S rRNA gene sequences of bacterial isolates from wild gypsy moths were deposited with accessions FJ013260 through FJ013269. Two representative 16S rRNA genes sequenced directly from this community were deposited with accessions FJ013270 and FJ013271.

Results

Bacterial communities from wild and lab-reared gypsy moth midguts have similar composition

We performed sequence analysis of 16S rRNA genes amplified from individual bacterial isolates from wild gypsy moths as well as a 16S rRNA gene library constructed from wild gypsy moth midguts. The members of the wild gypsy moth microbiota are consistent with previous results from lab-reared larvae (Table 1 and data not shown). Members of the Enterobacteriaceae and other Gammaproteobacteria were cultured from the wild gypsy moth larvae (Table 1). Culture-independent analysis of wild gypsy moth midguts revealed that 33 of 190 16S rRNA genes affiliated with *Pantoea agglomerans*, and 157 of 190 affiliated with *Enterococcus* sp. (data not shown), both of which are also found in the microbiota of lab-reared larvae (Broderick *et al.*, 2004). The bacterial community of wild gypsy moth larval midguts is therefore similar to that of lab-reared larvae.

Bacteria cultured from gypsy moth are resistant to common antibiotics

Among the 44 bacterial isolates from the midguts of lab-reared and wild-caught gypsy moth larvae, resistance was detected to 11 of the 12 antibiotics (no resistance to ciprofloxacin) (Table 1). Variations in antibiotic resistance profiles were observed among bacterial isolates of closely related species, and some of the levels of resistance were equal to or above the breakpoints for clinically significant resistance (NCCLS, 2004) (Table 1). Antibiotic resistance profiles were similar among bacteria isolated from lab-reared and wild-caught gypsy moth midguts (Table 1).

Metagenomic clones from the midgut microbial community confer a range of antibiotic resistance phenotypes

To determine whether any of the antibiotic resistance phenotypes observed in the culturable bacterial isolates from gypsy moth larvae were expressed in *E. coli*, metagenomic libraries were constructed from the pooled DNA of the bacterial isolates. The first library, GMC01, contained 8275 clones with an average insert size of 5.5 kb, and the second library, GMC02, contained 30,246 clones with an average insert size of 8.6 kb. These libraries captured over 300 Mb of DNA, which corresponds to at least sixty 5-Mb bacterial genomes. Forty-four isolates and therefore 44 genomes were pooled; thus, we estimate that each gene is represented at least once in the libraries, assuming evenness of representation.

Six unique clones that conferred tolerance to certain antibiotics were identified and confirmed (Table 2). Three clones (designated CRB01, CRB02, and CRB03) were originally isolated on media containing carbenicillin, two clones (designated ERY01 and ERY02) were isolated on media containing erythromycin, and one clone (designated CHL01) was isolated on media containing chloramphenicol. The resistance profiles of these clones were further characterized by broth microdilution MIC assays (Table 3). No ciprofloxacin- or kanamycin-resistant clones were isolated (data not shown). The results suggest that the clones encode three different types of antibiotic tolerance: one that is specific to erythromycin (in ERY01 and ERY02), one that is not specific to antibiotic class (in CHL01 and CRB03), and one that is specific to β-lactam antibiotics (in CRB01 and CRB02) (Table 3). Clones CRB01, CRB02, CRB03, and ERY01 were chosen for further genetic characterization.

The genes responsible for antibiotic resistance are diverse

Two of five ORFs in the clone ERY01 are responsible for the antibiotic resistance phenotype, as indicated by sequence analysis of transposon mutants (Fig. 1). The two genes encode homologs of SdeXY from *Serratia marcescens*, each with 95% amino acid identity. Consistent with the sequence analysis, PCR analysis showed *sdeX* to be present in both of the *S. marcescens* isolates and not in other isolates from the gypsy moth midguts (data not shown). SdeX and SdeY are proteins in the resistance-nodulation-cell division (RND) superfamily of multidrug resistance proteins.

The loss-of-phenotype transposon insertion mutants in CRB03 indicated that two putative ORFs are responsible for the observed phenotype, a *romA* homolog upstream of a *ramA* homolog (Fig. 1). We postulated that the insertions in the upstream gene were polar on *ramA* and therefore that *ramA* alone was responsible for resistance. This would be consistent with previous work showing that *ramA* from *Klebsiella pneumoniae*, but not *romA* as had been previously reported (Komatsu *et al.*, 1990), conferred multidrug resistance on *E. coli* (George *et al.*, 1995). To test this, we subcloned the *ramA* homolog and found that it alone increased resistance over the control *E. coli* strain (Table 3). The gypsy moth microbial community RamA is most similar to RamA from *K. pneumoniae* (86% amino acid identity). According to PCR analysis, the metagenomic *ramA* originated from all of the *Enterobacter* sp. isolates of the gypsy moth midgut (data not shown). In addition to the *ramA* homolog, another product was amplified with *ramA* primers from 17 of the isolates. Further experiments are necessary to determine whether this is a nonfunctional homolog of *ramA*.

CRB01 was subcloned, and sequence analysis revealed a gene whose deduced protein product has 60% amino acid identity and 72% amino acid similarity to BES-1, an extended-spectrum class A β-lactamase from *S. marcescens* isolated in several Brazilian hospitals (Bonnet *et al.*, 2000) (Fig. 1). CRB02 was found by PCR analysis to contain the same gene (denoted $bla_{\text{LRG-1}}$ as per the convention in the β-lactamase field), with 100% nucleotide identity (data not shown). PCR also indicated that the two *Erwinia* sp. isolates and the *Pseudomonas* sp. isolate from wild gypsy moths harbored $bla_{\text{LRG-1}}$. Both CRB01 and CRB02 were highly

Table 1. Minimum Inhibitory Concentration Values (μg/mL) of Culturable Bacterial Isolates from Gypsy Moth Midguts

Isolate[a]	*Source*[b]	*Crb*[c]	*Cft*	*Gnt*	*Ery*	*Kan*	*Str*	*Vnc*	*Cip*	*Chl*	*Rif*	*Nal*	*Tet*
Enterococcus faecalis	Artificial diet	64[d]	>**512**	16	<0.5	***128***	***256***	1	1	4	2	**256**	<0.5
E. faecalis	Larch	64	**512**	***64***	2	64	128	1	1	1	2	**256**	<0.5
E. faecalis	White Oak	64	>**512**	32	1	64	***256***	2	1	2	2	**256**	<0.5
E. faecalis	Willow	64	>**512**	32	1	64	64	1	1	2	1	**128**	<0.5
Enterococcus sp.	Aspen	64	**512**	8	<0.5	32	16	2	2	2	2	**256**	<0.5
Staphylococcus cohni	Artificial diet	***8***	***64***	<0.5	***8***	<0.5	<0.5	1	<0.5	2	<0.5	***128***	<0.5
S. cohni	Larch	2	16	<0.5	<0.5	<0.5	***4***	<0.5	<0.5	***8***	<0.5	32	***16***
S. cohni	Willow	***8***	***64***	<0.5	***4***	<0.5	<0.5	<0.5	<0.5	1	<0.5	64	<0.5
Staphylococcus lentus	Artificial diet	1	16	<0.5	<0.5	1	***4***	<0.5	1	***8***	<0.5	***128***	***8***
S. lentus	White Oak	4	32	<0.5	<0.5	<0.5	***4***	<0.5	<0.5	4	<0.5	32	***8***
S. lentus	Willow	2	32	<0.5	<0.5	1	2	<0.5	<0.5	4	<0.5	32	***16***
Staphylococcus xylosus	Artificial diet	4	32	<0.5	<0.5	***16***	***128***	1	<0.5	2	<0.5	***256***	***32***
S. xylosus	Larch	4	32	1	<0.5	***16***	***128***	1	<0.5	2	<0.5	***128***	***32***
S. xylosus	White Oak	***8***	32	<0.5	<0.5	<0.5	1	1	1	2	<0.5	64	<0.5
S. xylosus	Willow	***8***	32	<0.5	<0.5	<0.5	1	1	<0.5	2	<0.5	***256***	<0.5
Microbacterium sp.	Aspen	***64***	***512***	2	*1*	***32***	2	<0.5	1	1	<0.5	256	***4***
Microbacterium sp.	Larch	2	16	<0.5	<0.5	4	2	<0.5	2	2	<0.5	128	<0.5
Paenibacillus sp.	Larch	32	>**512**	<0.5	<0.5	1	>**512**	<0.5	<0.5	4	<0.5	4	<0.5
Sphingobacterium sp.	Aspen	**512**	16	8	8	>**512**	64	32	<0.5	4	<0.5	8	<0.5
Pseudomonas putida	Artificial diet	>**512**	8	***4***	8	2	***128***	**256**	<0.5	***256***	8	64	2
P. putida	Larch	>**512**	8	***4***	>***512***	***4***	***128***	>**512**	<0.5	***512***	16	64	4
P. putida	White Oak	>**512**	8	***4***	>***512***	2	***128***	>**512**	<0.5	***256***	16	32	2
P. putida	Willow	>**512**	8	***4***	>***512***	2	***128***	>**512**	<0.5	***256***	16	64	2
Pseudomonas sp.	Aspen	**512**	4	<0.5	>***512***	<0.5	16	>**512**	<0.5	16	8	32	1
Pseudomonas sp.	Larch	>**512**	8	<0.5	***512***	1	8	>**512**	<0.5	***128***	8	32	2
Pseudomonas sp.	Wild	**512**	8	1	***512***	***8***	8	**512**	<0.5	***128***	16	32	4
Enterobacter sp.	Artificial diet	8	<0.5	<0.5	8	2	2	**256**	<0.5	8	8	8	<0.5
Enterobacter sp.	Larch	16	<0.5	2	8	2	4	**512**	<0.5	8	4	4	<0.5
Enterobacter sp.	White Oak	8	<0.5	<0.5	16	2	4	**256**	<0.5	4	4	4	<0.5
Enterobacter sp.	Willow	16	<0.5	<0.5	8	2	1	**256**	<0.5	4	2	4	<0.5
Pantoea agglomerans	Artificial diet	2	<0.5	1	16	1	1	**512**	<0.5	2	8	1	<0.5
P. agglomerans	Larch	8	<0.5	<0.5	8	1	2	**256**	<0.5	1	2	1	<0.5
P. agglomerans	Willow	8	<0.5	1	16	1	2	**512**	<0.5	2	2	2	<0.5
P. agglomerans	Wild	***16***	<0.5	<0.5	8	1	1	**256**	<0.5	2	4	2	<0.5
P. agglomerans	Wild	8	<0.5	1	4	2	1	**128**	<0.5	4	8	4	<0.5
P. agglomerans	Wild	***16***	<0.5	2	16	***128***	4	**512**	<0.5	2	8	4	<0.5
P. agglomerans	Wild	8	<0.5	1	16	1	2	**256**	<0.5	2	8	2	<0.5
P. agglomerans	Wild	8	<0.5	1	***32***	2	1	**256**	<0.5	1	8	2	<0.5
P. agglomerans	Wild	8	***8***	1	8	1	2	**512**	<0.5	4	4	4	1
Pantoea sp.	Wild	***16***	<0.5	1	16	4	4	**512**	<0.5	4	8	4	1
Erwinia sp.	Wild	**512**	<0.5	1	32	2	1	>**512**	<0.5	2	16	***128***	1
Erwinia sp.	Wild	**512**	<0.5	1	32	2	***8***	>**512**	<0.5	2	16	2	1
Serratia marcesens	Aspen	8	<0.5	***4***	**256**	2	4	>**512**	<0.5	32	32	2	**128**
S. marcesens	Larch	8	<0.5	<0.5	**256**	2	4	>**512**	<0.5	32	32	1	**128**

[a]Isolate identification based on 16S rRNA sequencing.
[b]Source is either wild-caught (wild) or lab-reared gypsy moth larvae. If reared in the lab, the food is listed (eg: willow).
[c]Antibiotics are abbreviated as follows: Crb, carbenicillin; Cft, ceftazidime; Gnt, gentamicin; Ery, erythromycin; Kan, kanamycin; Str, streptomycin; Vnc, vancomycin; Cip, ciprofloxacin; Chl, chloramphenicol; Rif, rifampin; Nal, nalidixic acid; Tet, tetracycline.
[d]The data are coded as follows: baseline MIC as determined by comparing that of like gypsy moth isolates, black; high MIC in common with like gypsy moth isolates, boldfaced black; MIC at least twofold greater than that of like gypsy moth isolates, boldfaced black italics.

resistant to members of the penicillin structural class of β-lactams (amoxicillin, ampicillin, carbenicillin, and piperacillin), but not to the cephalosporin structural class (cefamandole, ceftazidime, cefoxitin, and cephalexin) (Table 4 and data not shown). One hallmark of extended-spectrum β-lactamases is that their activity is inhibited by commercially available β-lactamase inhibitors, such as clavulanate and tazobactam (Perez *et al.*, 2007). The activity of both CRB01 and a subclone of it containing the β-lactamase-encoding gene was abolished by clavulanate and tazobactam (Table 4). We have designated this β-lactamase LRG-1 (β-lactam resistance from gypsy moth midgut microbial community).

Table 2. Bacterial Cloning Strain, Plasmids, and Metagenomic Clones Described in This Work

Strain/plasmid	*Relevant characteristics*	*Source or reference*
E. coli strains and plasmids for cloning		
EPI300	*mcrA*, Δ(*mrr-hsdRMS-mcrBC*), *endA1*, *recA1*; high transformation efficiency of large DNA	Epicentre Biotechnologies
pCF430	Tet^r; broad-host-range vector	Newman and Fuqua, 1999
pET24a	Kan^r; cloning/expression vector	Novagen
Metagenomic clones in pCF430 from culturable bacteria of gypsy moth midguts		
ERY01	Ery^r; contains genes encoding multidrug resistance efflux pumps	This study; EU885955
ERY02	Ery^r	This study
CRB01	Crb^r; contains a gene encoding an extended-spectrum class A β-lactamase (ESBL)	This study; EU885954
CRB02	Crb^r; contains the same ESBL as CRB01 but has a different restriction pattern	This study
CRB03	Crb^r, Ery^r, Chl^r, and Nal^r; contains a gene encoding a transcriptional regulator	This study; EU885953
CHL01	Crb^r, Ery^r, Chl^r, and Nal^r	This study
Subclones in pET24a of selected active genes from the metagenome		
LRG1	β-lactamase from CRB01	This study
RAMA	transcriptional regulator from CRB03	This study

Antibiotics are abbreviated as follows: Tet, tetracycline; Kan, kanamycin; Ery, erythromycin; Crb, carbenicillin; Chl, chloramphenicol; Nal, nalidixic acid; ^{r}, resistant.

LRG-1 is related to the clinically relevant CTX-M family of class A β-lactamases

The predicted amino acid sequence of LRG-1 was analyzed phylogenetically in Bayesian and parsimony analyses. The resulting evolutionary trees revealed that LRG-1 is distantly related to all known β-lactamases (Fig. 2). It is most closely related to the β-lactamases ERP-1 (56% amino acid identity) and BES-1, the former of which is chromosomally encoded in *E. perscina* and the latter is plasmid-borne in *S. marcescens*. ERP-1 was the first extended-spectrum β-lactamase reported in a plant-associated enterobacterial species (Vimont *et al.*, 2002). The topology shows that LRG-1 did not arise from clinical β-lactamases, and suggests that BES-1 and LRG-1 have a common origin (Fig. 2).

Discussion

Our work demonstrates that the gypsy moth midgut microbial community harbors antibiotic-resistant bacteria and antibiotic resistance genes. The gypsy moth midgut community is therefore a potential source of new genes encoding antibiotic tolerance and clinically relevant levels of antibiotic resistance. In particular, the β-lactamase LRG-1 from the gypsy moth midgut metagenome was found to confer clinically relevant levels of resistance on *E. coli*. Extended-spectrum β-lactamases are increasingly common in the Enterobacteriaceae, including in isolates from livestock, companion animals, and wildlife (Costa *et al.*, 2006; Li *et al.*, 2007). Extended-spectrum β-lactamases hydrolyze both penicillins and cephalosporins, eliminating the effectiveness of a wider variety of β-lactams than do typical β-lactamases and causing higher mortality and morbidity rates in patients infected with pathogens carrying them than are caused by bacteria that do not produce these enzymes (Perez *et al.*, 2007). Several homologs of LRG-1 are in the CTX-M family of β-lactamases (Fig. 2), which is a group of plasmid-borne class A β-lactamases that has become the dominant extended-spectrum β-lactamases in pathogens isolated from humans (Bonnet, 2004). However, both the phylogenetic analysis and specificity of LRG-1 for penicillins suggest divergence between LRG-1 and the CTX-M family. LRG-1 originated in two bacterial species that were isolated from the midguts of wild gypsy moths, illustrating that insects might play a role in disseminating important antibiotic resistance genes. Future work will determine whether $bla_{\text{LRG-1}}$ is chromosomal or plasmid-borne in the gypsy moth microbiota.

The resistome (D'Costa *et al.*, 2006) of the gypsy moth midgut microbial community also contains genes, such as *sdeXY*, that encode efflux pump machinery of the RND family, which tend to have broad substrate specificity (Nikaido, 1998). The bacteria in the midgut community are confronted with a multitude of toxic compounds from the wide variety of plant species that the gypsy moth larvae consume, and therefore the pumps may be requisite to confront the chemically challenging environment in the midgut.

Table 3. MIC Values (μg/mL) of Clones and a Subclone from the Bacterial Metagenome of Gypsy Moth Midguts against Nine Antibiotics

Clone	*Crb*	*Cft*	*Gnt*	*Chl*	*Ery*	*Nal*	*Rif*
ERY01	8	0.5	1	2	256	4	16
ERY02	4	1	4	2	128	2	8
CRB01	>512	0.5	1	2	32	2	8
CRB02	>512	0.5	1	2	32	2	8
CRB03	64	4	1	16	256	16	16
CHL01	16	4	4	16	128	16	16
pCF430	4	0.5	1	2	32	2	8
RAMA	32	2	1	8	128	8	16
pET24a	2	1	1	2	32	2	4

Antibiotics are abbreviated as follows: Crb, carbenicillin; Cft, ceftazidime; Gnt, gentamicin; Chl, chloramphenicol; Ery, erythromycin; Nal, nalidixic acid; Rif, rifampin.

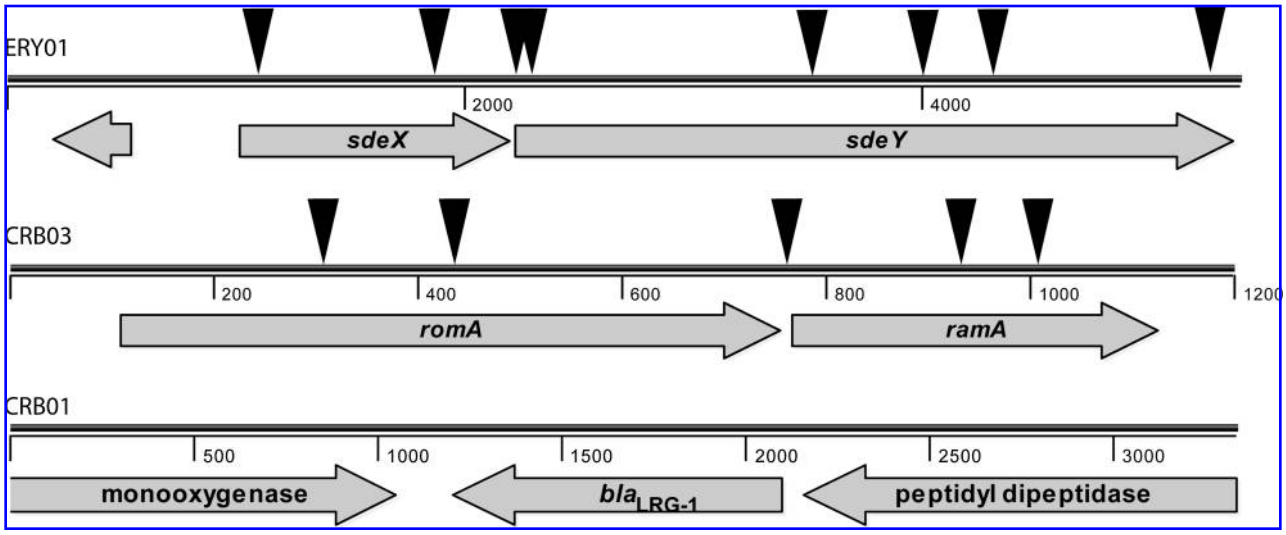

FIG. 1. ORF maps of antibiotic-resistant clones from the bacterial metagenome of the gypsy moth midgut. Only a portion of the insert sequence that contains the active gene is shown. Black triangles represent the location of transposon insertions that disrupted the resistance phenotype. The active gene in CRB01 was subcloned and not subjected to transposon mutagenesis. In ERY01, the gene product encoded by the unlabeled ORF is homologous to the TetR family of transcriptional regulators.

The resistome of the gypsy moth midgut community also revealed an AraC family transcriptional regulator that confers tolerance to numerous antibiotics on *E. coli*. The nearest homolog, *ramA*, was previously found in *K. pneumoniae*, *S. enterica*, and *E. aerogenes* (van der Straaten *et al.*, 2004b), which is consistent with our finding that the metagenomic *ramA* originated from the *Enterobacter* sp. of the gypsy moth microbiota. In these species and when overexpressed in *E. coli*, *ramA* confers multidrug resistance (George *et al.*, 1995; Chollet *et al.*, 2004; van der Straaten *et al.*, 2004a), although its precise role remains elusive. The *ramA* from the gypsy moth microbial community further illustrates the diversity of gene families that contribute to this antibiotic resistome.

Our analysis likely underestimates the extent of the gypsy moth midgut microbial community resistome. We screened for resistance only in isolates capable of producing turbid cultures

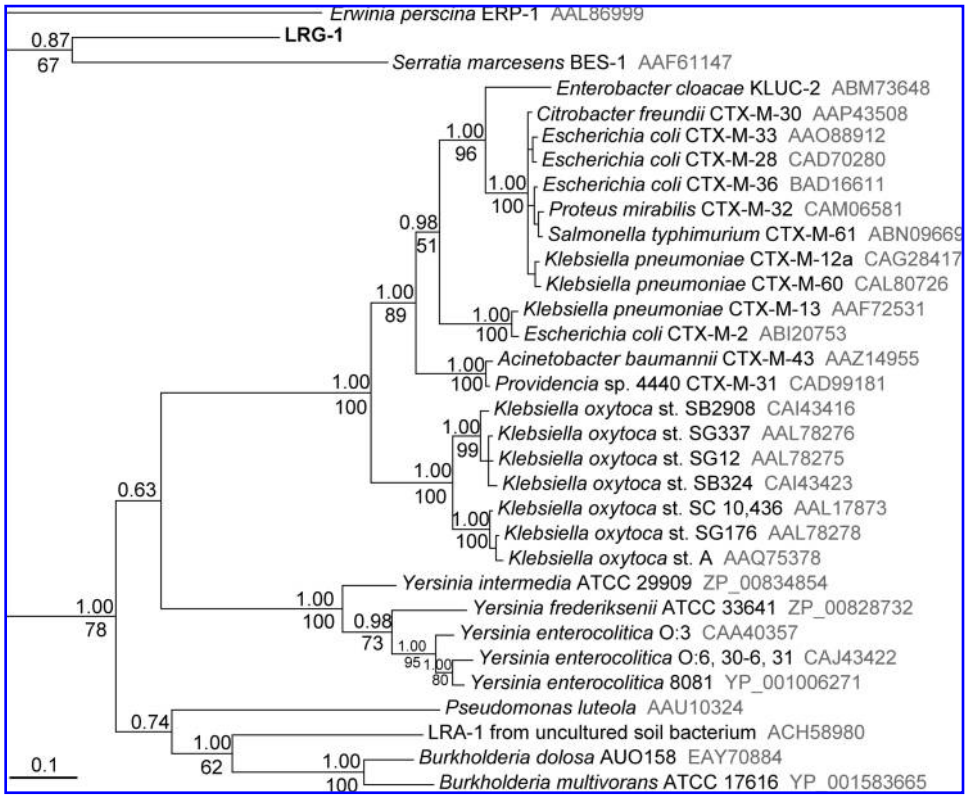

FIG. 2. Bayesian phylogenetic tree of aligned class A β-lactamase amino acid sequences. Bayesian posterior probabilities (200,000 MCMC generations, WAG + G model) are shown above the nodes, and bootstrap values based on parsimony analysis (1000 replicates) are below the nodes. Values are not reported for branches shorter than 0.01. Where the species on the tree are redundant, either the strain or β-lactamase name is also reported. Amino acid accession numbers are listed in gray. Scale bar = 0.1 changes/site.

Table 4. Inhibition of the (β-Lactamase from the Bacterial Metagenome of Gypsy Moth Midguts as Shown by MICs (μg/mL)

	β-*Lactams with and without inhibitor*					
	Amo			*Pip*		
Clone	–	*T*	*C*	–	*T*	*C*
CRB01	512	64	8	128	8	4
pCF430	8	4	2	4	4	4
LRG1	128	16	4	64	8	4
pET24a	4	4	4	4	4	4

Antibiotics and inhibitors are abbreviated as follows: Amo, amoxicillin; Pip, piperacillin; C, potassium clavulanate; T, tazobactam.

in Mueller–Hinton broth, and there may well be others that do not grow or express resistance in this medium. Further, the isolates were propagated without antibiotic selection, and therefore genes present on plasmids might have been lost. Although the 12 antibiotics tested in this study represent a broad range of drug classes and modes of action, resistance determinants specific for other antibiotics may well be present in the gypsy moth microbial community. It is also likely that the community contains resistance determinants that are not expressed in *E. coli*. Despite these limitations, functional metagenomics led us to diverse antibiotic resistance determinants: efflux pumps, transcriptional regulators, and enzymes that degrade antibiotics, thereby demonstrating that this is a streamlined method to identify resistance determinants in microbial communities. The similarity between the communities in lab-reared and wild-caught larvae suggests that the resistance determinants we isolated may be widespread in gypsy moth populations. In light of the size and density of gypsy moth populations, this work raises the concern that the bacteria or the resistance determinants from the gypsy moth gut might be transferred to other environments. Further work will determine whether the migration of resistance genes from insect guts or other environmental reservoirs to clinical settings presents a threat to human health.

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No competing financial interests exist.

References

Abascal, F., Zardoya, R., and Posada, D. (2005). ProtTest: selection of best-fit models of protein evolution. Bioinformatics **21,** 2104–2105.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J Mol Biol **215,** 403–410.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25,** 3389–3402.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev **59,** 143–169.

Bonnet, R. (2004). Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother **48,** 1–14.

Bonnet, R., Sampaio, J.L., Chanal, C., Sirot, D., De Champs, C., Viallard, J.L., Labia, R., and Sirot, J. (2000). A novel class A extended-spectrum beta-lactamase (BES-1) in *Serratia marcescens* isolated in Brazil. Antimicrob Agents Chemother **44,** 3061–3068.

Broderick, N.A., Raffa, K.F., Goodman, R.M., and Handelsman, J. (2004). Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. Appl Environ Microbiol **70,** 293–300.

Chollet, R., Chevalier, J., Bollet, C., Pages, J.M., and Davin-Regli, A. (2004). RamA is an alternate activator of the multidrug resistance cascade in *Enterobacter aerogenes*. Antimicrob Agents Chemother **48,** 2518–2523.

Costa, D., Poeta, P., Saenz, Y., Vinue, L., Rojo-Bezares, B., Jouini, A., Zarazaga, M., Rodrigues, J., and Torres, C. (2006). Detection of *Escherichia coli* harbouring extended-spectrum beta-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal. J Antimicrob Chemother **58,** 1311–1312.

D'Costa, V.M., McGrann, K.M., Hughes, D.W., and Wright, G.D. (2006). Sampling the antibiotic resistome. Science **311,** 374–377.

George, A.M., Hall, R.M., and Stokes, H.W. (1995). Multidrug-resistance in *Klebsiella pneumoniae*—a novel gene, RamA, confers a multidrug-resistance phenotype in *Escherichia coli*. Microbiology **141,** 1909–1920.

Gringorten, J.L., Crawford, D.N., and Harvey, W.R. (1993). High pH in the ectoperitrophic space of the larval lepidopteran midgut. J Exp Biol **183,** 353–359.

Hinnebusch, B.J., Rosso, M.L., Schwan, T.G., and Carniel, E. (2002). High-frequency conjugative transfer of antibiotic resistance genes to *Yersinia pestis* in the flea midgut. Mol Microbiol **46,** 349–354.

Huelsenbeck, J.P., and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17,** 754–755.

Kadavy, D.R., Hornby, J.M., Haverkost, T., and Nickerson, K.W. (2000). Natural antibiotic resistance of bacteria isolated from larvae of the oil fly, *Helaeomyia petrolei*. Appl Environ Microbiol **66,** 4615–4619.

Komatsu, T., Ohta, M., Kido, N., Arakawa, Y., Ito, H., Mizuno, T., and Kato, N. (1990). Molecular characterization of an *Enterobacter cloacae* gene (*romA*) which pleiotropically inhibits the expression of *Escherichia coli* outer membrane proteins. J Bacteriol **172,** 4082–4089.

Li, X.Z., Mehrotra, M., Ghimire, S., and Adewoye, L. (2007). Beta-lactam resistance and beta-lactamases in bacteria of animal origin. Vet Microbiol **121,** 197–214.

Macovei, L., and Zurek, L. (2006). Ecology of antibiotic resistance genes: characterization of enterococci from houseflies collected in food settings. Appl Environ Microbiol **72,** 4028–4035.

NCCLS. (2004). Performance standards for antimicrobial susceptibility testing; fourteenth informational supplement.

NCCLS document M100-S14. The National Committee for Clinical Laboratory Standards, Wayne, PA. **24,** 96–130.

Newman, J.R., and Fuqua, C. (1999). Broad-host-range expression vectors that carry the L-arabinose-inducible *Escherichia coli araBAD* promoter and the *araC* regulator. Gene **227,** 197–203.

Nikaido, H. (1998). Antibiotic resistance caused by gram-negative multidrug efflux pumps. Clin Infect Dis **27 Suppl 1,** S32–S41.

Pai, H.H., Chen, W.C., and Peng, C.F. (2004). Cockroaches as potential vectors of nosocomial infections. Infect Control Hosp Epidemiol **25,** 979–984.

Pai, H.H., Chen, W.C., and Peng, C.F. (2005). Isolation of bacteria with antibiotic resistance from household cockroaches (*Periplaneta americana* and *Blattella germanica*). Acta Trop **93,** 259–265.

Paterson, D.L. (2006). Resistance in gram-negative bacteria: Enterobacteriaceae. Am J Infect Control **34,** S20–S28.

Perez, F., Endimiani, A., Hujer, K.M., and Bonomo, R.A. (2007). The continuing challenge of ESBLs. Curr Opin Pharmacol **7,** 459–469.

Rahuma, N., Ghenghesh, K.S., Ben Aissa, R., and Elamaari, A. (2005). Carriage by the housefly (*Musca domestica*) of multiple-antibiotic-resistant bacteria that are potentially pathogenic to humans, in hospital and other urban environments in Misurata, Libya. Ann Trop Med Parasitol **99,** 795–802.

Rice, L.B. (2006). Antimicrobial resistance in gram-positive bacteria. Am J Infect Control **34,** S11–S19.

Riesenfeld, C.S., Schloss, P.D., and Handelsman, J. (2004). Metagenomics: genomic analysis of microbial communities. Annu Rev Genet **38,** 525–552.

Ronquist, F., and Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19,** 1572–1574.

Salyers, A.A., Gupta, A., and Wang, Y. (2004). Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol **12,** 412–416.

Sambrook, J., and Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Stoyenoff, J.L., Witter, J.A., and Montgomery, M.E. (1994). Nutritional indexes in the gypsy moth (*Lymantria dispar* (L)) under field conditions and host switching situations. Oecologia **97,** 158–170.

Swofford, D.L. (2003). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). **4.0b10**, available online at http://paup.csit.fsu.edu/.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **24,** 4876–4882.

Torsvik, V., Goksoyr, J., and Daae, F.L. (1990). High diversity in DNA of soil bacteria. Appl Environ Microbiol **56,** 782–787.

van der Straaten, T., Janssen, R., Mevius, D.J., and van Dissel, J.T. (2004a). *Salmonella* gene *rma* (*ramA*) and multiple-drug-resistant *Salmonella enterica* serovar *Typhimurium*. Antimicrob Agents Chemother **48,** 2292–2294.

van der Straaten, T., Zulianello, L., van Diepen, A., Granger, D.L., Janssen, R., and van Dissel, J.T. (2004b). *Salmonella enterica* serovar *typhimurium* RamA, intracellular oxidative stress response, and bacterial virulence. Infect Immun **72,** 996–1003.

Vasanthakumar, A.I., Handelsman, J., Schloss, P., Bauer, L., and Raffa, K.F. (2008). Gut microbiota of an invasive subcortical beetle, Agrilus planipennis Fairmaire, across various life stages. Environ Entomol **37,** 1344–1353.

Vimont, S., Poirel, L., Naas, T., and Nordmann, P. (2002). Identification of a chromosome-borne expanded-spectrum class a beta-lactamase from *Erwinia persicina*. Antimicrob Agents Chemother **46,** 3401–3405.

Whelan S., and Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol Biol Evol **18,** 691–699.

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